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Current knowledge on regulatory RNAs and their machineries in *Staphylococcus aureus*

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Staphylococcus aureus is one of the major human pathogens, which causes numerous community-associated and hospital-acquired infections. The regulation of the expression of numerous virulence factors is coordinated by complex interplays between two component systems, transcriptional regulatory proteins and regulatory RNAs. Recent studies have identified numerous novel RNAs comprising cis-acting regulatory RNAs, antisense RNAs, small non coding RNAs and small mRNAs encoding peptides. We present here several examples of RNAs regulating *S. aureus* pathogenicity and describe various aspects of antisense regulation.

Introduction

Staphylococcus aureus is a commensal bacterial species of human and a remarkable opportunistic pathogen, which is one of the main causes of hospital-acquired infections. *S. aureus* is responsible for a wide spectrum of human diseases ranging from minor skin infections to systemic infections such as sepsis and endocarditis.[1,2] One of the major concerns is the fact that the treatment of *S. aureus* diseases is hampered due to the emergence and spread of multi-drug resistant and hypervirulent strains.[3] Hence, determining the regulatory networks and their dynamics involved in fast adaptive responses and in virulence is a prerequisite to find alternative strategies to combat *S. aureus* infections.

A successful infection by *S. aureus* is caused by several immuno-evasive strategies, but also by the production of a large array of *S. aureus* virulence factors. These factors include surface proteins that are required for the adhesion and invasion of the host, exoproteins that are involved in host immune evasion, and toxins that disseminate in host tissues allowing the bacteria to acquire novel nutrients.[4-6] To be able to adapt to various environments, *S. aureus* has evolved a plethora of signaling pathways that coordinate the temporal expression of virulence genes in response to both environmental and host signals.[7] Protein-mediated transcriptional regulation is central in these regulatory circuits. Moreover, intricate and complex interactions are also occurring between multiple regulators such as two-component systems, regulatory proteins, peptide secondary metabolites, ribonucleases and RNAs.[7-9]

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Beside transcriptional control, regulation of mRNA translation and decay are major ways to rapidly modify gene expression in response to growth, perturbation of the environmental cues, stress and virulence.[9] Recent studies in *S. aureus* identified the machineries involved in mRNA decay and evidenced the role of several ribonucleases in the regulation of virulence gene expression. These enzymes have been recently considered as potential targets for therapeutic development against staphylococcal infections.[9] The stability of mRNAs is modulated by the activity of ribonucleases (RNases) but also by trans-acting factors such as small non coding RNAs (sRNAs) and RNA-binding proteins.[10-13] In 1993, the largest multi-functional regulatory RNA (RNAIII) was discovered to be the main intracellular effector of the quorum sensing system.[14] Later it was shown that RNAIII acts as an antisense RNA to regulate translation and decay of mRNA targets in a coordinated manner with the double-strand specific endoribonuclease III.[10,11,15,16] More recently, several studies revealed that the *S. aureus* genome likely encodes a huge diversity of sRNAs (reviewed in ref. 17 and 18), as demonstrated for many other bacteria.[19,20] These studies include computer software predictions coupled to expression studies,[21-24] microarrays,[13,25,26] cloning and conventional sequencing of small sized cDNA libraries[27] and high throughput sequencing approaches[28,29] (Lioliou et al. personal communication). More than 250 sRNA genes were discovered. Most of them are distributed all over the core genome while some of them are located in pathogenicity islands and plasmids. The sRNAs include cis-acting regulatory regions of mRNAs (the so-called riboswitches), cis-encoding antisense RNAs (asRNA) and non-coding RNAs (ncRNAs). In addition, mRNAs carrying small open reading frames (sORF) have been identified[29,30] and one of them was recently shown to express a small cytolytic peptide.[31] Although functional and mechanistic studies of sRNAs are still lagging behind, recent works show that sRNAs bring the missing links in the regulatory pathways that allow *S. aureus* to sense population density and various environmental changes, modify cell surface properties, adjust its metabolism during cell growth and regulate virulence gene expression (reviewed in ref. 17 and 18). More surprisingly, a recent genome-wide analysis combining high throughput sequencing and tiling arrays revealed a large number of discrete antisense RNAs indicative of pervasive transcription occurring along the *S. aureus* genome. In the same study, the role of RNase III in the degradation of sense/antisense duplexes was highlighted.[32] Hence,

ribonucleases and regulatory sRNAs are intimately linked in post-transcriptional regulation.

In this present review, we will describe several novel and representative mechanisms exploited by regulatory RNAs and their machineries to control gene expression in *S. aureus*. A particular focus will be on regulatory RNAs involved in virulence.

Cis-acting Regions of mRNAs Regulate Metabolic Pathways Essential for Bacterial Growth

In bacteria, the 5' untranslated regions (5'-UTR) of numerous mRNAs have evolved dedicated regulatory sites which act as direct sensors of the physical and metabolic states of the cell.[33-36] Some of the most widespread cis-acting elements of mRNAs are the so-called riboswitches, which regulate expression of downstream genes in response to elevated concentrations of specific metabolites.[37] Elegant biochemical, genetic and structural studies reveal the mechanisms by which riboswitches achieve a strict specificity to small ligands to regulate expression of the downstream gene.[37-39] Impressive structural studies of the most widespread riboswitch classes show that each ligand binds to a dedicated and conserved cleft formed by a compact three-dimensional folding of the 5' UTR (reviewed in ref. 39). This specific interaction often induces mRNA conformational changes that have direct consequences on the expression of the following coding sequence.[40] Based on sequence and structure conservation,[41,42] 15 riboswitches were mapped on *S. aureus* genome. Until now, 7 operons and 33 genes are expected to be under the control of riboswitches specific for S-adenosylmethionine (SAM), thiamine pyrophosphate (TPP), flavin mononucleotide (FMN), lysine, glycine, guanine, 7-aminomethyl-7-deazaguanine (preQ1) and glucosamine-6-phosphate (Glc-6P).[23,24,27-29,43] For many of them, alternative basepairings can form either an anti-terminator hairpin or a Rho-independent terminator suggesting that binding of the metabolite (SAM, TPP, lysine, glycine, preQ, FMN) to the aptamer domain would induce premature transcription arrest.[18] A highly conserved yybP motif was predicted to regulate transcription termination/anti-termination of SA0878 encoding a transporter protein.[18,44] This simple but highly common motif in bacteria has been often associated with genes involved in controlling the cellular pH.[44] Interestingly, recent works show that alkali pH enhanced the duration of RNA polymerase pauses on the nascent *alx* mRNA in *E. coli* and promoted the folding of an active pH responsive element to enhance translation.[45] This would certainly mean that either the RNA polymerase or the RNA might sense the proton or hydroxide ions. Such a pH effect has not yet been demonstrated in *S. aureus* although it was reported that pH variation affected virulence factor production.[46]

Two riboswitches, which are expected to recognize Glc-6P and FMN, might regulate their downstream genes, *glmS* and SA1316, respectively, by different mechanisms. In Gram-positive bacteria, Glc-6P acts as a co-factor of the regulatory domain of *glmS* mRNA and confers a catalytic activity to the 5'UTR.[47] The crystal structure of *glmS* regulatory region has shown that the active site is pre-folded and that the ribozyme activity is only promoted by Glc-6P binding.[48,49] This initial cleavage might lead to rapid degradation of *glmS* mRNA.[50] The conservation of the catalytic site strongly suggests that the Glc-6P-induced cleavage is also preserved in *S. aureus*. For the FMN riboswitch, which controls expression of the hypothetical protein SA1316, alternative pairings are predicted to regulate the accessibility of the ribosome binding site. Hence, FMN binding would stabilize the formation of a hairpin structure sequestering the SD sequence to inhibit translation initiation.[18] Two unusual mechanisms involving SAM riboswitches have been described in Gram-positive bacteria. In *Clostridium acetobutylicum,* a SAM riboswitch acts as antisense RNA to control the expression of *ubiG* operon via transcriptional interference.[51] In *Listeria monocytogenes*, a SAM riboswitch was shown to regulate in trans the translation of *prfA* mRNA encoding a transcriptional activator of expression of virulence factors, highlighting a link between bacterial virulence and nutrient availability.[52] Although such mechanisms are not yet described in *S. aureus*, transcriptomic analysis has shown that short transcripts comprising the whole regulatory regions were detected most likely due to premature transcription termination.[28,29] Furthermore, additional large 5'UTRs and 3'UTRs have been recently identified suggesting that they might carry specific regulatory domains.[28,29]

All these riboswitches regulate the synthesis of essential proteins involved in amino acid transport and biosynthesis, as well as co-factor and nucleotide biosynthesis. As these regulatory elements evolved specific binding pockets suitable for the recognition of small molecules, they have been considered as tractable targets for anti-microbial compounds.[33,53] Recent work has successfully led to the design of non-metabolizable agonistic molecules targeting the guanine riboswitch in *S. aureus* based on the crystal structure of the riboswitch.[54] A pyrimidine derivative compound, which was able to bind efficiently the guanine riboswitch, constitutively switches off the expression of the essential *guaA* gene encoding GMP synthase. This compound shows bactericidal activity against *S. aureus* and reduced infection in mice. As atomic structures are solved for numerous classes of riboswitches,[39] they offer new opportunities to design novel chemical compounds with anti-microbial activities.[37]

Intricate Interactions between Regulatory Proteins and RNAs in Quorum Sensing System

The quorum sensing system, which senses the population density, has multiple functions in *S. aureus* physiology and pathogenesis. It is aimed not only to respond to environmental changes but also to regulate virulence gene expression.[55,56] While the system coordinates the temporal expression of numerous virulence factors, strategies have been developed to interfere with virulence and prevent the rapid appearance of drug resistance.[7] Briefly, the system is composed of two divergent transcripts, RNAII encoding a quorum sensing cassette and a two-component system and RNAIII encoding hemolysin delta (**Fig. 1**). The quorum sensing cassette produces the autoinducer peptide AIP which upon a threshold concentration, activates the membrane kinase AgrC and the response regulator AgrA through a phosphorylation mechanism. Studies performed with methicillin resistant

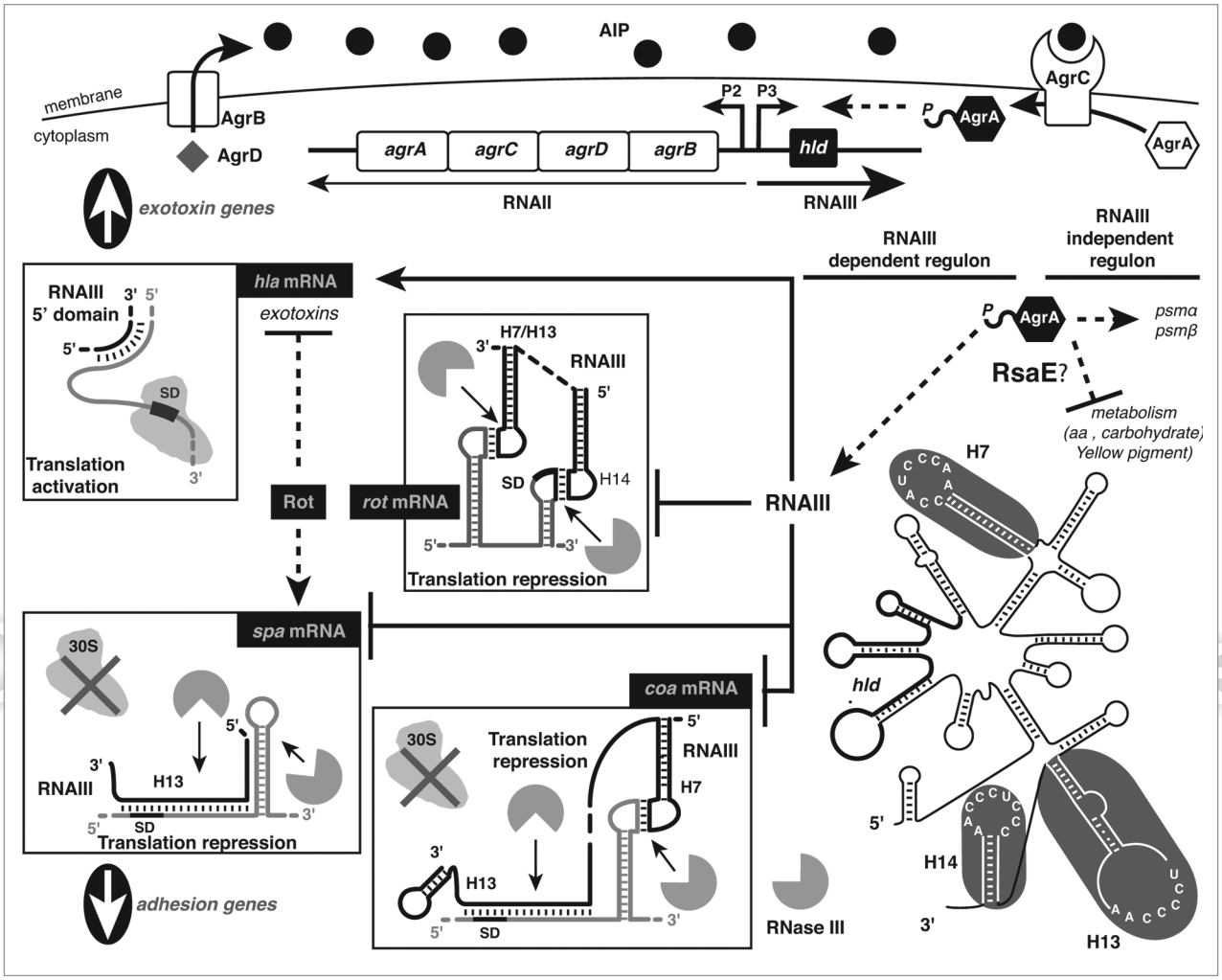

Figure 1. The *agr* system and its two main intracellular effectors AgrA and RNAIII. The quorum-sensing circuit is shown at the top: it involves the auto-inducing peptide (AIP, black circle) matured and exported by AgrB. AIP activates the AgrC/AgrA two-component system. The *agr* system regulates the expression of multiple genes in an RNAIII-independent manner via AgrA and in an RNAIII-dependent manner. RNAIII-independent regulation: AgrA activates the synthesis of several peptides by binding to their promoter regions (Phenol soluble modulin, PSM) and represses metabolic enzymes by an unknown mechanism. Repression might occur via the expression of a small non-coding RNA, RsaE, which is enhanced by AgrA. RsaE regulates enzymes of the central metabolism.[23,28] RNAIII-dependent regulation: the secondary structure of RNAIII and three of the hairpin domains containing a redundant UCCC motif are colored in gray. RNAIII encodes hemolysin delta (*hld*). The 3′ non coding region of *hld* which contains the three redundant hairpin loops binds to the ribosome binding sites of *coa* mRNA encoding coagulase, *rot* mRNA encoding repressor of toxins Rot, and *spa* mRNA encoding protein A. In all cases, RNAIII prevents the binding of the 30S ribosomal subunit to inhibit translation and recruits the endoribonuclease III (RNase III) to initiate rapid degradation. The 5′ non coding region of *hld* binds to *hla* mRNA to facilitate ribosome binding and to activate translation. Dashed bars and dashed arrows are for transcriptional regulation while black bars and arrows are for post-transcriptional regulation. Bars are for repression and arrows for activation. The data are taken from previous works.[10,15,16,57,59]

S. aureus strains show that AgrA directly activates the expression of the adjacent RNAIII and the synthesis of several cytolytic PSM peptides while it represses the synthesis of metabolic enzymes and of staphyloxanthin biosynthesis by an unknown mechanism.[57] Interestingly, recent observations show that AgrA enhances expression of the sRNA RsaE, which in turn represses the synthesis of enzymes involved in the TCA cycle at the post-transcriptional level.[23,28] Whether AgrA activates expression of other sRNAs to repress expression of RNAIII-independent target genes is still an open question. RNAIII was also shown to coordinate the temporal expression of numerous accessory factors.[6,14] Hence the two effectors of the quorum sensing system, namely AgrA and RNAIII, might reflect a stepwise evolution in the lifestyle of *S. aureus*, in order to establish its pathogenicity.[57]

RNAIII is one of the fascinating regulatory and structured mRNAs that regulates multiple targets involved in virulence and peptidoglycan metabolism.[6,17,58] The non-coding parts of RNAIII are the regulatory domains, which control gene expression at the post-transcriptional level via an antisense mechanism. The 5'UTR of *hld* binds to the leader region of *hla* mRNA to facilitate ribosome recruitment (**Fig. 1** and ref. 59) while the large 3' UTR is acting primarily as a repressor domain. The 3'UTR is characterized by three redundant hairpin structures with a conserved C-rich sequence located in the apical loops. This motif is

often used to bind G-rich sequences in mRNAs located primarily in the ribosome binding sites (**Fig. 1**). Although the topologies of the RNAIII-mRNA duplexes are different, they all efficiently prevent ribosome binding and recruit the double-strand specific endoribonuclease III (RNase III), which initiates the rapid degradation of the repressed mRNAs.[10,15,16] These mRNAs encode adhesin factors (protein A, coagulase, SA1000) and the transcriptional repressor of toxins, Rot. Through the regulation of Rot, RNAIII indirectly activates the transcription of exotoxins and represses the transcription of adhesins such as protein A.[10,11] Hence, RNAIII regulates the synthesis of *spa* and *hla* mRNAs at two different levels involving feed-forward regulatory loops (**Fig. 1**). Such a double regulation ensures that the repression is irreversible and provides a rapid induction of exotoxins.[60,61] Recent work suggested that part of the coding sequence of *hld* could bind to *map* mRNA encoding the major histocompatibility complex class II analogous protein to activate translation[62] suggesting that activation of the *map* mRNA translation may interfere with *hld* translation. In addition, RNAIII represses several hydrolases and amidases involved in the metabolism of peptidoglycan and hence it may contribute to the cell wall integrity at high cell density[10,56] (Lioliou et al. personal communication). Regulation of peptidoglycan metabolism might be the common function of RNAIII in all staphylococcal species including *S. epidermidis*. Apart from RNase III, is there another protein that facilitates the RNAIII functions? In contrast to Gram-negative bacteria, the Sm-like Hfq protein has no detectable effect neither on RNAIII-target mRNA complex formation, on RNAIII-dependent regulation in vivo, nor on the RNAIII stability.[10,23,63]

Many questions await further experimental works: is the structure of RNAIII sufficient for its regulatory functions? Does RNAIII target other mRNAs encoding regulatory proteins? Does the translation of *hld* affect regulation? Does RNAIII act in concert with other sRNAs? Do other sRNAs contribute to regulation of virulence gene expression? The importance of RNAIII/AgrA for *S. aureus* pathogenesis has also been the subject of debate. The vast majority of clinical isolates from acute infections express RNAIII.[64] However, the level of RNAIII may vary considerably among clinical isolates[65] and the pattern of proteins regulated by RNAIII is not conserved in all isolates.[57,66] Recent study suggested that *agr* mutations, which are often found in methicillin-resistant strains, might be an adaptation of the pathogen within the infected host but conversely this loss of function might be counter-selective outside the host.[67] The regulators involved in the modulation of RNAIII expression or its stability are not known. Another future task would be to understand how modulation of RNAIII concentration may affect its regulatory circuits and what could be the advantages for *S. aureus* to develop variations in the pattern of virulence gene expression.

S. aureus Acquires Novel sRNAs through Mobile Elements

Mobile genetic elements play essential roles in genome evolution since they favor the acquisition of novel functions that have conferred to *S. aureus* a wide range of adaptive processes for survival in its hosts.[68-70] These elements include prophages, transposons, plasmids and pathogenicity islands (SaPI). Although several major virulence factors are encoded on the core genome, others are carried on SaPIs such as the superantigens and several enterotoxins, implying that these factors have been transferred horizontally. Another type of mobile element, the so-called staphylococcal chromosome cassette (SCCmec), confers resistance to methicillin and other antibiotics to *S. aureus* strains.[70] Mobile elements and extra-chromosomal elements are tightly controlled so that they do not compromise the integrity of the host. Among the regulatory factors, short antisense RNAs (asRNA) were the first regulatory RNAs discovered to control transposition, plasmid replication, partition and conjugation in the 80s years (reviewed in ref. 71 and 72). In 1989, the first asRNA discovered in *S. aureus* was shown to control the replication of pT181 plasmid.[73] Later, several ncRNAs, asRNAs and mRNAs encoding small peptides have been identified in SaPIs, prophages and SCCmec.[21,27-29,74,75] Mechanistic and functional studies on some of these RNAs revealed unexpected features that are summarized below.

An sRNA mediates inter-relations between pathogenicity islands and the core genome. Several sRNAs, named SprA-G for small pathogenicity island RNA, were identified in SaPIs.[21,31,76] The fact that the sRNAs are expressed from SaPIs, which were horizontally acquired, does not preclude that the RNA will regulate target genes located within the same locus. An example is provided by SprD, a ncRNA expressed from PIϕ.[21,29] SprD was shown to repress translation initiation of *sbi* mRNA encoding an immune-evasion molecule, a gene located on the core genome.[76] SprD contains four hairpin structures and the third one interacts with the ribosome binding site of the *sbi* mRNA to form a long duplex of 40 base pairs interrupted by bulged nucleotides. The interaction is sufficient to prevent translation initiation in vivo and has no effect on the half-life of *sbi* mRNA. As for *S. aureus* RNAIII, SprD-dependent repression takes place independently of the Sm-like Hfq protein.[76] Strikingly, *sbi* mRNA strongly decreased at the stationary phase of growth while the yield of SprD remained almost unchanged suggesting that an additional level of regulation takes place independently of SprD. Whether this downregulation is *agr*-dependent and affects transcription or mRNA degradation have still to be addressed. SprD contributed to *S. aureus* diseases in a mouse model, but this effect was not linked to the repression of Sbi production, indicating that SprD might regulate the synthesis of other proteins that play major roles during the host infection.[76] This work suggested that the regulatory functions of SprD have evolved to mediate interconnections between the SaPI and the core genome, in order to regulate virulence gene expression.

Antisense RNAs with different properties. Several short and structured asRNAs have been identified to regulate plasmid replication,[73] and to repress the expression of transposase[28,29] or the synthesis of small cytolytic peptides.[31] These sRNAs are short, highly structured and contain regions that are fully complementary to their target mRNAs since they are transcribed in the opposite direction. Analysis of the mechanism of action for several of them reveals unusual binding pathways that are directly

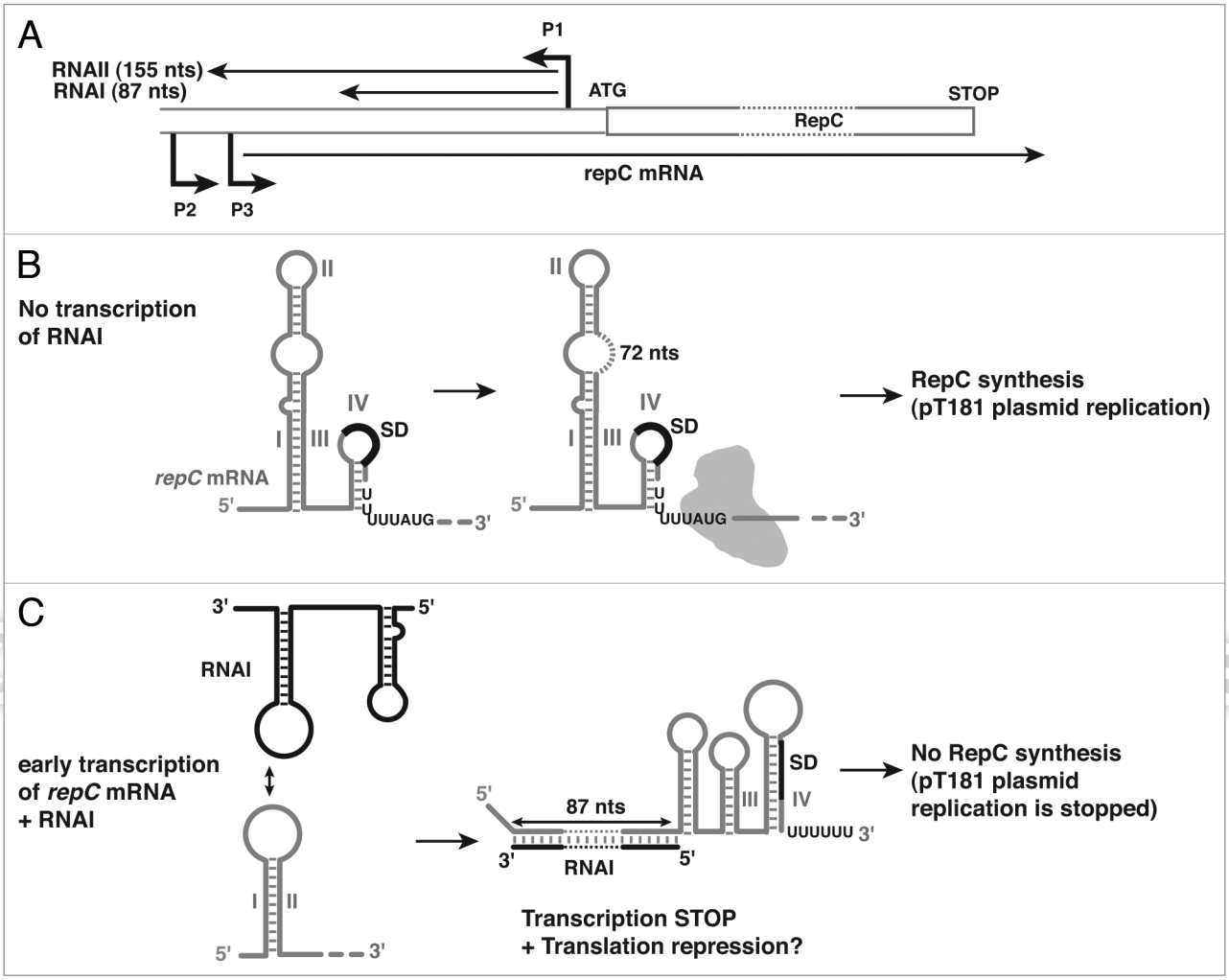

Figure 2. Antisense regulation of plasmid pT181 replication. (A) Genetic organization of pT181 plasmid and its control region. RNAI is the antisense RNA. (B) Schematic secondary structure model of *repC* mRNA leader region as proposed by Novick et al.[73] The formation of the large helical domain formed by helices I and III favors the formation of an anti-terminator hairpin to favor the transcription of the whole mRNA. In this structure, the Shine and Dalgarno sequence (SD) and the initiation codon are available for translation. (C) The antisense RNAI traps a transient hairpin structure of *repC* mRNA during transcription, and the formation of the RNAI-mRNA duplex stabilizes a Rho-independent terminator to arrest transcription. RepC synthesis is thus abolished.

dependent on the structures of the mRNA and the asRNA. Novick and collaborators were the first to describe how the antisense RNAI causes transcriptional attenuation of the mRNA encoding the rate-limiting replication RepC protein of plasmid pT181 [73] (**Fig. 2A**), a mechanism often used in plasmids of Gram-positive bacteria.[71,72] This plasmid confers to the bacteria several antibiotic resistances. The mechanism is as follows. The *repC* mRNA can potentially adopt two distinct conformations in which an anti-terminator or a terminator of transcription can be alternatively form (**Fig. 2A and B**). In the absence of RNAI, the nascent *repC* transcript forms preferentially into a conformation that permits transcription throughout the *repC* mRNA. As the consequence, translation can occur since the Shine and Dalgarno sequence (SD) is accessible to recruit the ribosome (**Fig. 2A**). In the presence of RNAI, the asRNA binds to a hairpin structure of *repC* mRNA that is transiently formed during transcription. The initial binding probably occurs via a loop-loop interaction involving several G-C pairings that is subsequently converted into a duplex of 87 base pairs (**Fig. 2C**). The formed complex modifies the folding process of the mRNA during transcription so that a Rho-independent terminator structure is stabilized just upstream the initiation codon, resulting in a premature termination of transcription. In addition, this hairpin structure sequesters the SD sequence of *repC*, which might block the access of the ribosome (**Fig. 2C**). The mechanism relies on specific properties. The antisense RNAI has to bind to the 5'UTR of *repC* mRNA within a short time window to be effective so that the mRNA folding pathway during transcription can be modified. The formation of the sense-antisense duplex is indeed a very fast and productive process that is a direct consequence of specific interactions between designed three-dimensional structures of the interacting RNAs, a feature that is generally common to short asRNAs regulating essential processes of extra-chromosomal elements.[71]

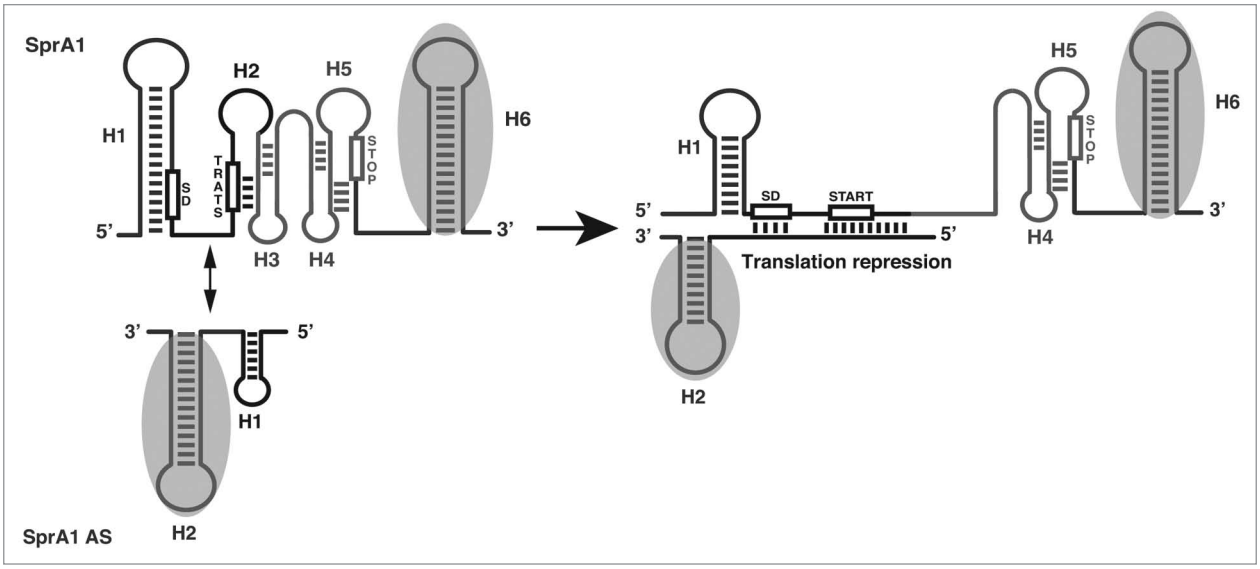

Figure 3. Antisense regulation of the synthesis of a small cytolytic peptide encoded by SprA1. The secondary structures of SprA1 and of the antisense RNA SprA1 AS are from Sayed et al. SprA1 contains two pseudoknot structures H2 and H5. SprA1 is a mRNA which encodes a small cytolytic peptide. The 5′ end of SprA1 AS forms imperfect base pairings with the ribosome binding site of SprA1 and unfolds the pseudoknot structure H2 to prevent translation. SD is for Shine and Dalgarno, START is for the initiation codon, and STOP for the stop codon. The fully complementary regions of SprA1 and SprA1 AS (surrounded in gray) do not interact. The model of repression mechanism is from Sayed et al.

A recent work has revisited and questioned the definition of the active region of a short and structured asRNA.[31] Among the SprA-G sRNAs, two of them, SprA1 and SprG were shown to be expressed together with their asRNAs, SprA1-AS and SprF, respectively.[21,29] It was previously predicted that these two RNA pairs form type I toxin-antitoxin modules in which SprA1 and SprG would encode small hydrophobic peptides.[77] The SprA1/SprA1-AS module is found in several copies in the core genome and plasmids. SprA1 was proposed to be a multifunctional RNA since its 3' end could potentially base pair with the 3'-UTRs of three mRNA targets.[21] The peptide, resulting from the translation of SprA1 sORF, has a lytic activity on human erythrocytes and displays an antimicrobial activity against Gram-positive and Gram-negative bacteria.[31] The SprA1 encoded peptide shares physicochemical properties with *S. aureus* PSM, which are also amphipathic α-helical peptides.[57,74] An sRNA encoding a PSM was recently identified within the SCC-mec cassette, and this PSM has pro-inflammatory and cytolytic activities, and has significant impact on immune evasion and disease.[74] PSM genes are also present in multi-copies and RNAIII encodes one of them. However, an asRNA against mRNA expressing a PSM was not demonstrated so far. Both SprA1 and SprA1-AS are constitutively expressed and the yield of the asRNA is significantly higher than that of SprA1.[31] The structure of SprA1 is characterized by two pseudoknot motifs in which the ribosome binding site is partly imbedded but this conformation does not hinder the formation of the ribosomal initiation complex.[31] Structure probing combined with mutational analysis shows that the short SprA1-AS binds to the ribosome binding site of SprA1 through imperfect pairings to prevent translation of the peptide (**Fig. 3**). Unexpectedly the interacting region does not involve the 3' end of SprA1-AS, which is fully complementary to the 3' end of SprA1 and is dispensable in vivo. Instead, the active region is located in its 5' part that is partially complementary to the ribosome binding site of SprA1 (**Fig. 3**). The reason for this unexpected observation lies in the structures of the two RNAs. The fully complementary regions of both RNAs correspond to Rho-independent terminators, which are highly stable hairpin motifs terminated by four G-C base pairs. Such stable structures probably do not favor the rapid formation of stable intermolecular pairings. Instead, the 5' unpaired region of SprA1-AS may be more appropriate to promote fast and early binding with the connecting loop L2 of the pseudoknot structure of SprA1 (**Fig. 3**), a mechanism used by sRNAs of Gram-negative bacteria.[78] Again, Hfq was not found associated with the antisense RNA regulation. In light of this study, the structures and the sequences of both the sRNA and the target must be taken into account to identify the regulatory sites involved in antisense regulation. In addition, this study adds further evidence that *S. aureus* expresses a large variety of peptides.[6,30,31,70,75,77] Interestingly, the PSM peptides are controlled by AgrA[74] as is the quorum sensing AIP peptide while two other hypothetical peptides were activated by the alternative σB factor required for stress adaptation, antibiotic resistance and virulence.[30] Whether these short mRNAs regulate gene expression as it was shown for *S. aureus* RNAIII remained to be studied. Elucidation of their functions, structure and mechanisms of action is certainly another area of interest.

Is there a Specific Machinery Associated with sRNA Function?

Little information yet exists on *S. aureus* RNA-binding proteins (RNA chaperone, RNA helicase, enzymes, post-transcriptional regulators…) that might be associated with sRNA regulation.

In contrast to Gram-negative bacteria, all the sRNAs studied so far which regulate gene expression though mRNA binding do not require the Sm-like Hfq protein (see below). However, there is a growing list of works showing that mRNA stability is a highly controlled process in *S. aureus* that allows the pathogen to respond to environmental variations, nutrient availability, growth rate and cell density (reviewed in ref. 9, 12 and 79). Among the proteins that alter mRNA turnover, the pleiotropic transcriptional regulatory protein SarA was unexpectedly identified.[13] Whether SarA binds to RNA or indirectly affects mRNA degradation through the activation of sRNAs are open questions. Moreover, the roles of several ribonucleases in *S. aureus* virulence have been appreciated,[9] and among these enzymes, RNase III has been clearly identified as a major partner in sRNA regulation.[10,15,16,32] Some of the recent data are discussed below.

The controversy concerning *S. aureus* Hfq protein. Hfq is the well-known Sm-like RNA chaperone protein whose function has been deeply studied in Gram-negative bacteria (reviewed in ref. 80). Hfq is able to bind sRNAs and mRNAs,[81,82] promotes RNA conformational changes favoring the binding of sRNAs to their target mRNAs, stabilizes sRNAs against degradation and mediates transcription antitermination at Rho-dependent terminators through binding to Rho.[83] The first crystal structure of Hfq bound to a A(U)5G sequence was solved in *S. aureus* revealing a homohexameric ring made of two distinct faces where the AU5G oligonucleotide binds to the proximal face.[84] Comparison with the structure of *E. coli* Hfq protein reveals that the overall structure in both distant bacteria is very similar but significant differences in the repartition of charges have been observed.[85,86] This is particularly true for the region connecting the proximal and distal faces, which is strongly negative in *S. aureus* and conversely strongly positive in *E. coli*.[87] Such differences might have some consequences on the RNA recognition and the role of Hfq in the formation of mRNA-sRNA complexes. Works in *E. coli* and *S. aureus* show that the proximal site would be dedicated to the recognition of internal A/U rich sequences of sRNAs while the distal site would preferentially recognize A-rich sequences.[87] More recent studies described that the proximal face of *E. coli* Hfq has a strong preference for the Rho-independent terminator U-tail sequences of sRNAs and has a particularly high affinity for the 3'OH end of a U6 oligonucleotide.[88,89] This specific recognition of the 3' end of sRNA by Hfq plays a direct role in the stabilization of sRNA.[88] Comparison of *S. aureus* Hfq-A(U)5G and *E. coli* Hfq-U6 reveals that the conformation of the RNA backbone is very different in both structures where it is highly constrained in *E. coli* allowing a specific recognition of the 3' terminal hydrogen bond of the RNA by Hfq.[89] These differences are most likely linked to the nature of the sequences of both oligonucleotides rather than the sequences of Hfq itself.[89]

Although *S. aureus* Hfq binds to RNA, its role in sRNA-dependent regulation remains obscure in contrast to enterobacteria.[90] Deletion of the *hfq* gene in various strain backgrounds (RN6390, COL, Newman) shows that Hfq is not involved in stress response, antibiotic resistance and virulence.[11,63] *S. aureus* Hfq binds tightly to RNAIII in vitro but has no detectable effect on RNAIII-target mRNAs complex formation.[10,15] A similar behavior was found for several other sRNAs targeting mRNAs.[23,31,63,76] Moreover Hfq is not involved in the stability/turnover of sRNAs.[23,30,31,63,76] As many of the experimentally studied sRNAs interact rapidly with their target mRNAs in vitro and form stable complexes sufficient to prevent ribosome binding, they might preclude the need for Hfq. It was also proposed that there is an apparent link between the dispensability of Hfq, the GC content of the bacterial genomes and the stability of the formed sRNA-mRNAs.[91] This apparent paradox between the absence of in vivo effect of Hfq and the ability of the protein to bind RNA in vitro was explained by the fact that Hfq is not produced in RN6390/COL strains.[11] The reason why Hfq cannot be detected has not been yet investigated although it was suggested that mutations in the promoter region led to a significant decrease of *hfq* transcription.[11] Interestingly, a recent work[92] showed that Hfq is detected only in several clinical strains and in the 8325-4 strain but not in COL and RN6390, two laboratory-adapted strains.[11,63] In 8325-4, where Hfq is expressed, its deletion decreases pathogenicity in a murine peritonitis infection model.[92] Specifically, the deletion of Hfq deregulates the expression of 116 genes, many of them being associated to virulence.[92] More specifically, deletion of *hfq* causes increased synthesis of the surface carotenoid pigment and decreased synthesis of exoproteases.[92] In light of this latter work, the behavior of *S. aureus* (8325-4 strain) cells was analyzed in a low-fluid-shear environment where the bacteria initiated a novel attachment-independent biofilm phenotype.[93] These cells displayed slower growth and attenuated virulence characteristics, such as decreased carotenoid production, increased susceptibility to oxidative stress and reduced survival in blood. Microarray profiling of these cells showed alterations in several metabolic pathways, and unexpectedly, expression of the *hfq* gene was strongly decreased. This study suggested that *S. aureus* 8325-4 strain responds to a low-fluid-shear environment by initiating a biofilm/colonization phenotype with decreased virulence characteristics that could be in part attributed to *hfq*.[93] These two studies show that Hfq impacts stress response and pathogenesis when it is expressed. Conversely, in other strains, Hfq function may have been superseded by other factors and hence this riboregulator has been downsized by mutational loss of its promoter. Hence, many questions have now to be addressed more specifically: what are the functions and the mechanisms of action of Hfq in strains expressing the protein? Does Hfq promote the formation of some of sRNA-mRNA pairs? Does *S. aureus* Hfq bind to RNAs similarly than the *E. coli* Hfq protein? Are there functional Hfq analogs particular/specific to strains that do not express Hfq? How does regulation occur in strains deficient in Hfq? What would be the advantages of expressing (or not) Hfq in *S. aureus*?

Interestingly, a recent work has shown that the conserved and ubiquitous metallo-protein SMc01113 in *Sinorhizobium meliloti*, ortholog to *E. coli* YbeY, alters the accumulation of sRNAs and their mRNA targets in a way similar to Hfq.[94] Although these effects might be indirect since the protein has been associated with the maturation of the ribosome at high temperature,[95,96] the protein shares intriguing structural similarities with the MID domain of eukaryotic AGO proteins.[97] This protein might be part of the bacterial sRNA pathway,[94] and although It is found

in several *S. aureus* strains, its function has not been studied yet. In *B. subtilis*, it was shown that three small basic proteins act as RNA chaperones of the sRNA FsrA, to promote degradation of mRNAs encoding iron-dependent proteins under conditions of iron deprivation.[98] Obviously, the use of genetic screens and/or purification of ribonucleoprotein particles might help to identify the protein co-factors of *S. aureus* sRNAs.

The RNase III as a major player of RNA-dependent regulation. *S. aureus* RNase III belongs to a large class of ubiquitous enzymes which cleave double-stranded RNA (dsRNA) to generate short RNA duplexes ended by 2 nt 3'-overhang. In eukaryotes, the equivalent enzymes are involved in biogenesis of siRNA/miRNA in higher organisms.[99] RNase III is a highly conserved Mg^{2+}-dependent endoribonuclease among bacteria and consists of a catalytic and a dsRNA binding domain and functions as a homodimer (reviewed in ref. 100). *S. aureus* RNase III is capable of recognizing and cleaving a variety of structures such as imperfect duplexes, helices interrupted by bulged residues and loop-loop interaction.[101] Besides the well-known function in the maturation of large rRNAs, a recent study shows that the *E. coli* RNase III has a widespread role in cellular mRNA processing and contributes to the turnover of many sRNAs thus affecting indirectly the transcriptome.[102] In *S. aureus*, the enzyme acts as a co-factor of the quorum-sensing dependent RNAIII to coordinate the repression of several mRNA targets encoding protein A, coagulase and the repressor of toxins Rot.[10,15,101] Binding of RNAIII to its mRNA targets facilitates the decay by RNase III thus rendering the repression irreversible. Given the fact that in Gram-positive bacteria, sRNAs form rather long duplexes with target mRNAs,[10,103] the role of RNase III in antisense regulation is probably more widespread than previously expected. A recent study based on deep sequencing to analyze short RNA fractions of *S. aureus* has revealed a large collection of 22 nt long RNA fragments generated by RNase III digestion of sense/antisense transcripts all over the chromosome.[32] More than 75% of the mRNAs were subjected to specific RNase III processing as a result of antisense regulation. Deletion of RNase III reduced significantly the amount of short RNA fragments and concomitantly accumulation of low levels of antisense transcripts were visualized.[32] These data are indicative of antisense transcription all over the genome and this pervasive transcription is hidden due to RNase III processing of sense/antisense transcripts. This activity of RNase III can be considered as an RNA quality control mechanism to remove efficiently transcriptional noise. Interestingly, the involvement of RNase III in this novel post-transcriptional process appears to be restricted to Gram-positive bacteria.[32] Moreover, several asRNAs were transcribed in a σB-dependent manner, and the levels of the sense RNA was regulated accordingly by RNase III.[32] Therefore under specific conditions, the levels of asRNAs can reach a threshold concentration so that the mRNA yield can be modulated.[32] Besides antisense regulation, this study calls to the question, how many targets can RNase III recognize? Based on in vivo immunoprecipitation of wild type or cleavage-defective mutant RNase III followed by deep sequencing, we have recently identified a large number of RNA targets (Lioliou et al. personal communication). In addition to rRNA processing and mRNA turnover, the enzyme has been associated with novel functions such as processing of mRNAs with overlapping 5'UTRs, maturation process stabilizing the mRNA, sRNA and asRNA-dependent regulation. Finally, a recent work shows that inactivation of the *rnc* gene in the 8325-4 strain decreased the synthesis of extracellular toxins due to a destabilization of RNAIII and secY2 mRNA, one component of the accessory secretory pathway.[104] The effect of RNase III on RNAIII levels was not observed in RN6390,[15] but as previously described some significant differences between 8325-4 and RN6390 strains were observed although they originated from the same parental strain.[105] Deletion of *rnc* in 8325-4 strain resulted in a less virulent strain compared with the isogenic wild type strain in a murine peritonitis model[104] while the *rnc* mutant had no effect on cell growth.[15,104] Taken together, all these studies show that RNase III is a global player of RNA-dependent regulation in *S. aureus*, which has consequences on the regulation of the synthesis of virulence factors.

The impact of other ribonucleases in *S. aureus* pathogenesis and sRNA regulation. Other enzymes than RNase III are also expected to be associated with the sRNA-dependent regulation. In *E. coli*, the endoribonuclease RNase E and the phosphorolytic exoribonuclease PNPase are both required for sRNA-dependent regulation.[106,107] These two enzymes are part of a multi-enzymatic complex, the so-called degradosome, which also contains the RNA helicase RhlB and the glycolytic enzyme enolase (reviewed in ref. 108). Although there is no homolog of RNase E in Gram-positive bacteria,[109] several enzymes were proposed to be organized into a multi-enzymatic complex in *S. aureus*[26] and *B. subtilis*.[110,111] Using various strategies, networks of interactions have been identified between two glycolytic enzymes (enolase and phosphofructokinase), the DEAD-box RNA helicase CshA and four RNases, namely RNase J1, RNase J2, RNase Y and PNPase.[26,110,112] The two *B. subtilis* enzymes RNase J1/J2 are endowed with a dual activity of an endoribonuclease and a 5'-3' exoribonuclease[113] while RNase Y, is the functional equivalent of RNase E and cleaves mRNA at unpaired U/A rich sequences.[114,115] Hence, *S. aureus* and *B. subtilis* share the same components and three of them (enolase, PNPase, DEAD-box RNA helicase) are also conserved in Gram-negative bacteria. More surprisingly, the protein subunit of RNase P, RnpA, is associated with CshA in *S. aureus* and this interaction takes place in *B. subtilis* too.[26] Although the degradosome-like is conserved in Gram-positive bacteria, the interaction networks between the components diverged slightly.[26] In *S. aureus*, the two proteins, CshA and enolase, are central since CshA binds to enolase, Pflk, RNase Y, RNase J1 and RpnA while enolase recognizes PNPase, RNase Y and CshA (**Fig. 4**). However, this work does not provide information on the stoichiometry between the different partners, nor if these interactions happen in the same complex. The interactions might be highly dynamic and sub-populations of the degradosome could occur depending on the subcellular localization of the RNases or of the RNA. It is intriguing that glycolytic enzymes are also part of the degradosome raising the question about the functional links between metabolic enzymes and ribonucleases. The glycolytic enzymes are known to respond to both glycolytic and TCA cycle intermediates, and are thus

sensors of nutritional stress. Hence, it was proposed that they might coordinate the action of RNases under energy limiting conditions.[116] Interestingly, functional relationships between metabolic adaptation to nutritional status, mRNA stability regulation and virulence factor production have been well described (Somerville and Proctor, 2009), and at least three RNase components of the degradosome-like may play a role in virulence and stress adaptation, namely PNPase, RNase Y and RpnA.[26] Disruption of the *S. aureus pnpA* gene induced a mild change in mRNA turnover, however, cells became sensitive to cold shock.[12] These data suggested that mRNA turnover plays an important role in response to stress and changes of the environmental cues. *S. aureus cvfA* gene encodes RNase Y, which contains a transmembrane domain, a KH RNA-binding domain and a metal-dependent phosphorylase (HD) domain.[117,118] RNaseY has been first described as an enzyme endowed with a phosphodiesterase activity carrying by its HD domain, which is required for hemolytic activity and virulence in mice and silkworm infection models.[118] However, works performed in *B. subtilis*, have shown that RNase Y is an endoribonuclease with a preference for 5' end monophosphorylated mRNA and cleaves unpaired U/A rich sequences in structured regulatory regions of mRNAs such as the SAM-riboswitch.[114] Interestingly enough, the localization of RNase Y at the membrane is essential in vivo indicating that subcellular localization is required for the turnover of a subset of mRNAs.[115] Although the target genes of RNase Y are not yet defined in *S. aureus*, deletion of *cvfA* represses the transcription of the *agr* operon resulting in the repression of exotoxin genes, and in the accumulation of protein A.[117] The third protein RpnA, which is the co-factor of RNase P, is an essential protein most likely due to its role in tRNA maturation.[119] Unexpectedly RpnA, which adopts an aß-fold structure,[120] exhibits a ribonuclease activity in vitro against rRNAs and mRNAs. This activity was not demonstrated in vivo although a high number of mRNAs were more stable in *rnpA* deficient cells. A specific compound (RNPA1000), which inhibited the ribonuclease activity of RnpA in vitro, was recently selected. It decreased growth of Gram-positive bacteria, prevent biofilm formation and impaired pathogenesis in a murine acute model of *S. aureus* infection.[121] Thus RNA decay machinery appears to be an appropriate target for the design of anti-microbial therapeutics. Considering the development of high throughput methods, one could expect to gain knowledge on the whole set of RNA targets of ribonucleases. This will be of importance in order to get a more complete picture of the complexity of the RNA-dependent regulatory networks and of the role of these enzymes in sRNA regulation in this facultative pathogen.

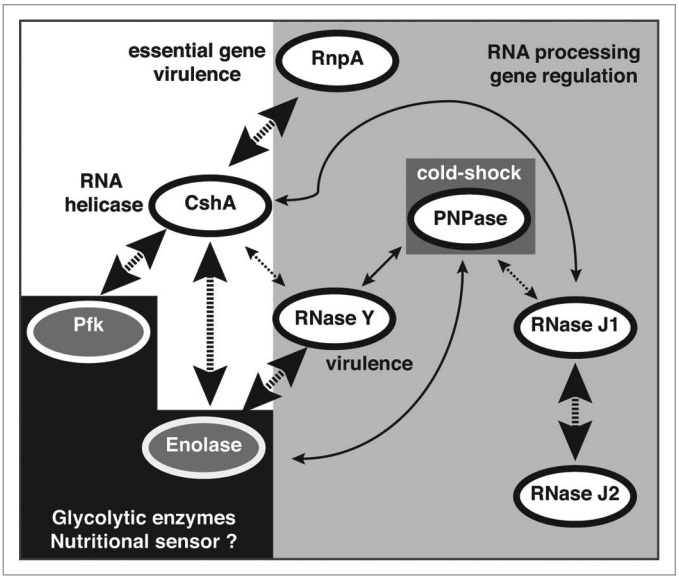

Figure 4. Networks of interactions involving several ribonucleases. The interactions have been demonstrated in vivo using the double hybrid approach.[26] Thickness of the arrows represents the strength of the interaction. Full arrows represent interactions that are specific of *S. aureus* whereas dashed arrows are for interactions conserved in *S. aureus* and *B. subtilis*. The data are adapted from Roux et al.[26] PNPase is for polynucleotide phosphorylase, CshA for DEAD box RNA helicase, Pfk for phosphofructokinase, RnpA for the protein component of RNase P and RNase for Ribonuclease. Specific functions of proteins in virulence, stress response, gene expression and RNA metabolism are given. The different gray colors denote similar functions for the protein partners.

Perspectives

This review gives a brief glimpse on the functions of some of the regulatory RNAs from *S. aureus*. However, we are still far to get a full appreciation of the sRNA functions and of the complex and intermingled interactions occurring between sRNAs and regulatory proteins to regulate gene expression. The sRNAs for which the functions have been determined show how they interconnect metabolism, stress adaptation and virulence, and how they mediate crosstalks between pathogenicity islands and the core genome. Deep mechanistic studies show that sRNAs acting as antisense RNA bind to their mRNA targets with very efficient ways that are dependent on designed three-dimensional structures to favor efficient initial contacts followed by rapid propagation of intermolecular base pairings. Thus, the specificity of antisense regulation not only relies on sequence complementarities but also on specific structure motifs of RNAs that prevent or favor the pairings. Continued studies of individual sRNAs and their machineries should certainly unravel unexpected functions and regulatory mechanisms, such as novel cis-acting regulatory elements, sRNA targeting enzymes, sRNA involved in antibiotic resistance.

One major point is the fact that it is difficult to generalize the impact of sRNA regulation in *S. aureus* due to great variability between the strains. For instance, detailed comparison of strains expressing or not Hfq would certainly be of importance to monitor the gain of function. Another challenge is to understand the contribution of *S. aureus* sRNAs in the human context and to gain knowledge about the roles of sRNAs and their co-factors during human colonization and infection.

Note

Several works showed that Staphylococcus aureus Hfq failed to substitute Escherichia coli Hfq (Vecerek B, Rajkowitsch, L.,

Sonnleitner E., Schroeder R. & Blasi U. The C-terminal domain of E. coli Hfq is required for regulation. Nucleic Acids Research 2007; 36: 133-143) and Salmonella typhimurium Hfq (Rochat T, Bouloc P, Yang Q, Bossi L & Figueroa-Bossi N. Lack of interchangeability of Hfq-like proteins. Biochimie 2012; in press) in sRNA-mediated regulation in vivo."

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